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Acute growth inhibition & toxicity analysis of nano-polystyrene spheres on *Raphidocelis subcapitata*

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ABSTRACT

Micro/nano-plastics (MNPs) have been found within many environments and organisms including humans, making them a significant and growing concern. Initial research into the potential detrimental effects these MNPs both from acute and chronic exposure has been ongoing but still requires substantially more data to clarify. This research presents the response of nano-polystyrene (NPS) on *Raphidocelis subcapitata*, a freshwater alga, under an existing acute toxicity test along with additional analytical techniques to try identifying possible sources of toxicity. *R. subcapitata* cells were exposed for 72 h to a concentration range of 0–100 mg/l NPS. Growth Inhibition (GI) testing showed the *R. subcapitata* demonstrated statistically distinct reductions in growth over 72 h at all NPS exposure concentrations while not suffering culture collapse. By the 100 mg/l NPS exposure the *R. subcapitata* has suffered almost a 33.7% reduction in cell concentration after 72 h compared to control samples. Confocal imaging showed the NPS wasn't permeating into the algal plasma membrane or individual organelles but agglomerated onto the algal cell wall. The agglomeration was irregular but increased in total surface area covered as NPS concentration increases. UV–Vis fluorimetry testing produced a linear response of emission intensities to algae exposed to the 0–100 mg/l range of NPS. However, comparisons of emission intensity values of algae exposed to NPS to emission intensities of pure NPS at identical concentrations showed consistent intensity reduction. This response further indicated NPS agglomerating within the media and onto the alga cells seen from confocal imaging. Finally, Raman spectroscopy on *R. subcapitata* attempted to distinguish the key 1001 cm^{-1} peak or other crucial identifier peaks of polystyrene from overall Raman spectra. This was not successful as emissions from algal component (e.g. phenylaniline) completely suppressed the signal region.

1. Introduction

The environment has seen an increase in the levels of plastic waste added to it over the many decades since plastics were regularly introduced. From the sheer quantity and variety of plastic types and structures, and the numerous ways they are disposed of, plastics have become a universal issue in the environment (Chae and An, 2018; Chow et al., 2017; Swift, 2015). Those same plastics disposed after usage in landfills become worn-down over years from wind and rainfall, released from polymer-containing fabrics in clothing or material and chemical degradation processes, turning into plastic fragments that can be washed away (He et al., 2019; Su et al., 2019; Sundt, 2018; Zambrano et al., 2019). Plastics are also being released from polymer fabrics such as clothing which often remain in their polymer structure but become easy picked up and made air-borne (Gasperi et al., 2018; Prata, 2018a; Syafei et al., 2019). Existing research found bulk plastics within the environmental underwent degradation such as mechanical weathering or UV degradation, including eventual fragmentation into micro-scales which retained their base monomer structure and properties but could risk being detrimental due to their increased surface area and size permeability (Barnes et al., 2009; Brandon et al., 2016; Lambert and Wagner, 2016; Weinstein et al., 2016; Wu et al., 2019). These micro/nano-plastics (MNPs) have been a persistent concern over the last few decades over the rise of these produced or degraded plastics that exist in micro & nano scales are rising in the environment (Jiang, 2018; Peng et al., 2017; Zhang et al., 2019). Their impact that these MNP products released into the environment has remained broadly unclear, with a need for research to determine the potential acute and chronic

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consequences.

One area of existing concern is the presence of MNPs found throughout freshwater bodies as the result of the degraded waste plastics left within the river systems of the world (Leslie et al., 2017; Nizzetto et al., 2016; Rodrigues et al., 2018b; Strungaru et al., 2019; Triebskorn et al., 2018). MNPs of all plastics formats and structures have been found in freshwater systems across the world, both suspended in the water itself and in the sediment layer under and around the water (Free et al., 2014; Lahens et al., 2018; Mahon et al., 2017; Mani et al., 2015; Rodrigues et al., 2018a; Wang et al., 2017, 2018). These reports showed MNP contamination suspended in freshwater bodies is quite varied, with a Vietnamese rivers getting up to 519 particles per litre but water systems in Europe getting results of only up to 6.5 particles per litre in Irish drinking water and 53 μg/L in Portuguese river water. It has also been shown that wastewater processing plants often fail to fully prevent the emission of MNP from contaminated waste material, including a literature review by Habib et al. on 42 wastewater treatment plants (WWTP) from various studies showing a range of 0.01–35.6% MNP passing though the WWTP, with studies indicating polystyrene microparticles being removed at rates above 93% (Habib et al., 2020; Pivokonsky et al., 2018; Prata, 2018b; Talvitie et al., 2017; Weithmann et al., 2018; Ziajahromi et al., 2017). These numerous research articles noting detections and permeation already demonstrated the build-up of these MNPs within freshwater organisms, noting their potential for wide-spread harm. At the same time the presence of microplastics within water-bodies could impact humans from both direct contact with rivers and lakes, or from the consumption of water organisms and materials already contaminated with these plastics (Catarino et al., 2018; Galloway, 2015; Iñiguez et al., 2017; Karbalaei et al., 2018). However it is important to state this research article was not attempting to utilize concentrations of MNP matching real-world ranges, as the focus was to run an acute toxicity test model at levels that could induce discernible impacts on the algae in the short testing period.

One of the fundamental organisms within this food-chain that is liable to the MNP contamination and thus upline contamination are the algal group. Algae represent one of the most fundamental parts of the food-chain, acting as the primary producer that converts basic minerals within water-sources through photosynthesis into basic compounds of nutrition that ultimately feed primary consumers in the water (Braun and Schagerl, 2010; Chapman, 2013; Kastovsky et al., 2019; Lee, 2018). Being one of the most universal organisms for sustenance within the aquatic world, algae are of vital importance to every other organism which consumes them and in turn all species of secondary consumers all the way up to humans (Alexander et al., 2016; Anbumani and Kakkar, 2018; Avio et al., 2017; Ferreira et al., 2019; Santana et al., 2017). It is vital to consider whether the increased presence of MNPs within the environment will contribute to a distinct loss or contamination of these algal cells. There is existing evidence for algal toxicity testing, with even reports of plastic structures in the micro/nano scale producing a mixed response of impairment with aquatic organisms (Karami et al., 2016; Lei et al., 2018; Murphy and Quinn, 2018; Niels Nyholm and Kallqvist, 1989; Tosetto et al., 2017; Varó et al., 2019). A distinct area of concern was surface adsorption or agglomeration of MNP particles to the algal cell walls, several studies of which has been shown to block photosynthesis and growth inhibition (Bergami et al., 2017; Bhattacharya et al., 2010; Nolte et al., 2017; Zhang et al., 2017). For our research, we are examining the impact of a fluorescently tagged nano-plastic (100 nm polystyrene spheres with 440 nm excitation wavelength) when exposed in a freshwater environment containing a select algal culture *Raphidocelis subcapitata*. These algae are a "sickle" shaped freshwater micro-alga (formally *Pseudokirchneriella subcapitata*) that grow up to 15 μm in length and have been used in toxicity testing to represent freshwater algae (Rocha et al., 2017; Sohn et al., 2015; Tuominen et al., 2013).

The testing was conducted using OECD standardized testing No. 201 with minor adaptation to more accurately represent a real-world media (OECD, 2011). The OECD test was conducted in a rotating incubator with growth lamps and constant mild oscillations in a river-media substitute to best replicate a real freshwater body in motion. This investigation was run to determine if mg/L concentrations of nano-polystyrene (NPS) particles caused detrimental effects to algae in an acute test model rather than a μg/L concentration of MNP replicating current environmental levels. The analysis focused on exposing healthy algae to NPS and examining if there was a resulting decrease in the growth rate when compared to control algal samples. The research will also analysed fluorescence imaging and quantification techniques to examine the locations of NPS contamination and judge if contamination on cells increased with NPS concentration. Confocal analysis would examine whether NPS was present on algal cells, and if so whether it merely coated their surface or permeated into their cell organelles. The UV–Vis fluorimetry would be used to enhance the confocal data by comparing emission intensities from identical concentrations of NPS placed in either pure media or media containing algae. Should NPS in algae-containing media show consistent reductions in intensity compared to NPS in pure media, this would further indicate the agglomeration of NPS to the surface of the algal cells. Finally, Raman analysis would examine if spectroscopic evidence could be produced on non-processed algal samples on whether fingerprint signal of polystyrene could be discerned in exposed samples. These combined methods of analysis will thus determine whether the NPS is adsorbed by the algae and inducing toxic responses and/or potentially block replication and nutrient uptake by coating the exterior of the algal cells.

2. Materials & methods

2.1. Materials

2.1.1. Nano-polystyrene spheres (NPS)

In order to represent the micro/nano-plastic, a polystyrene particle was used on the higher size of the nanoscale (100 ± 10 nm diameter) containing a tagging dye. The particle was Thermofisher Scientific Fluoro-Max G100 polystyrene microsphere which had a specialized green fluorescent dye called Firefli, with an excitation/emission range of 468/508 nm, abbreviated to NPS (Thermo Scientific, 2011). This nano-plastic is ideal as it is suspended within pure water, along with being available in a concentrate stock of 1% solid, equalling to 10,000 mg/l. The most crucial factor behind choosing these particles was the combination of fluorescent dye assist in NPS location analysis without the main concern of dye leaching. The Firefli dye has been integrated into the styrene chains of the polystyrene, which provides a clear and precise fluorescence that will demonstrate the exact positions of NPS particles. The stability of the dye within the particle and their leaching potential were already conducted by a TUDublin student and were shown to be very stable (Dorney, 2013). As such the NPS can act both as a suitable comparator for expected nano-plastics that manufacturers could produce and risk release into the environment along with additional fluorescence detection capabilities. To prevent surface ionization and agglomeration the NPS contained trace levels of a proprietary surfactant produced by Thermofisher Scientific. Attempts were made to receive the individual surfactant or the MSDS details, however following communication with Thermofisher the only details given was that the surfactant was structurally and toxicologically similar to sodium dodecyl sulphate (SDS) and it was at a ratio of 0.2 μg of surfactant to every 1 mg of NPS.

Since SDS is considered an aquatic toxin it was important to examine the risk factor to the algal sample, however we had no access to the actual surfactant. As such there was no ability to conduct an accurate surfactant control sample for analysis however research articles were scrutinized to find crucial risk factors (LC50, LOEC, etc.) of SDS to the test algae. The literature review found firstly an MSDS stating the lowest observable effect concentration (LOEC) on *P. subcapitata* exposed to SDS of 2.68 mg/l after 6 days (144 h) (Sigma-Aldrich, 2018). An MSDS reported range of EC50 values from 3.59 to 117 mg/l after 96 h with

toxicity increasing when samples were left static (which our samples won't be)(Thermo Scientific, 2019). Following this, two research literature sources found the SDS IC50 of 36.58 mg/l and 36.51 mg/l respectively for *R subcapitata* (Feng et al., 2019; Liwarska-Bizukojc et al., 2005). Our most concentrate NPS exposure was 100 mg/l, as such the highest volume of surfactant released into the medium (0.2 μg/mg surfactant per NPS) would be 20 μg/l. The conclusion from the combination of these literature assessments demonstrates that for tests results, even at the worst response (3.59 mg/l EC50), our samples are exposed to only 0.56% of that value. This clearly demonstrates the SDS is going to have a very negligible effect on our algal samples during testing assuming the Thermofisher proprietary surfactant reacts similarly to SDS as indicated. Additionally, the acute toxicity test we conducted should include the risk posed by potential manufactured nano-plastics in the future, with the surfactant potentially playing a part in their toxicity. This means that it was important that the responses represented the combined issues of NPS, surfactant, and any chemical that becomes adhered to the NPS after the possible loss of surfactant.

2.1.2. Algal culture

Raphidocelis subcapitata is a micro-sized (15–50 μm^2 surface area) freshwater algae. In a healthy form they are a sickle or "C" shape making them highly definable for physiological change or damage (Nygaard et al., 1986; Suzuki et al., 2018). This *R. subcapitata* has been widely utilized in ecotoxicology because of its rapid response to even low-level aquatic contaminants. The *R. subcapitata* was supplied by the City Analytics laboratory, Shannon, Ireland and maintained in DIT FOCAS Aquarium lab. Cultures of algae were stored within 750 ml glass conical flasks suspended in 250 ml Jaworski Media (JM). The algae were kept homogenous by storing them in a New Brunswick INNOVA 44R Incubator Shaker. The incubator maintained a constant 22 ± 2 °C temperature with a 75-rpm oscillation and 16h/8h day/night cycle using combined white and photosynthetic light sources. The *R. subcapitata* was sub-cultured weekly, diluted to 50,000 cells/ml with JM and excess algal media disposed of. JM is a regularly utilized algal suspension medium designed by Prof Schlösser that contains a variety of vitamins, minerals, and ionic and metallic salts (Naha et al., 2011; Schlösser, 1982). JM was produced well in advance of usage to ensure it goes through a series of purification and balancing phases. The JM was stored in 1L Duran bottles and checked for pH balance and hardness to ensure it met required conditions, before being placed into the aquarium lab to acclimatize to the environmental conditions. Once a bottle was required, an oxygenator was inserted and run for at least 3 h to ensure saturated dissolved oxygen and the removal of any ammonia.

2.2. Characterisation of NPS

2.2.1. Particle size confirmation and stability testing (DLS)

As previously stated, a source of concern was that the components of JM could strip the surfactant and ionise the surface of the NPS. Prior studies have already shown the increase of this surface charge and the potential to agglomerate the particles along with attracting contaminants from the media (Bhattacharya et al., 2010; Hüffer et al., 2018, 2017; Klein, 2015; Liu et al., 2016; Nolte et al., 2017). The NPS were analysed using dynamic light scattering (Zetasizer Malvern Nano-series) to ensure particle size was consistent with manufacturer stated values (100 ± 10 nm diameter). Six samples were prepared, three samples of 20 mg/l NPS with 30 ml de-ionised (DI) water and a further three samples of NPS in 30 ml JM. This was conducted by pipetting stock NPS (10,000 mg/l) into individual 50 ml volumetric flasks before pipetting in the required media. The NPS solutions were sealed into the flasks and left in the 20 ± 2 °C sealed incubation shaker at 75-rpm with a 16h/8h light/dark cycle for up to 72 h. This would ensure the impacts were representative of the future experimental set-up for algae toxicity testing, along with complete homogenous suspension of the NPS within their media due to constant oscillation. Following every 24 h, 2 ml of media was pipetted into three separate 5 ml cuvettes before being placed in the Zetasizer. Scans were run with 3 ml samples in 5 ml cuvettes refractive index 1.33 for polystyrene with water as dispersant from 0.1 to 10,000 nm diameter particle detection to match pre-stated NPS characteristics. The core analysis was to determine if the simple process of diluting down the NPS media could increase possible instabilities, either from particle degradation or agglomeration. The NPS was tested using both DI and JM to evaluate and compare the stability of NPS within both solutions. Should the JM minerals induce further agglomeration or disintegration with the NPS, the DLS spectrum should display a reduced intensity but a spread size compared to DI water samples.

2.2.2. UV–visible fluorimetry (UV–Vis)

Examinations for the fluorescence emission from the dye in the NPS spheres was conducted with UV–Vis fluorescence analysis (Spectra-Max M Microplate Readers). A dilution was prepared of 100 mg/l NPS with DI water by pipetting stock NPS into a glass 50 ml volumetric flasks. The media was then homogenised using mild sonication (using a Branson 2510) run at 40Hz frequency submerged in 25 °C water for 5 s. Once homogenised into the water, 3 ml of the dilute NPS media was pipetted into separate 5 ml quartz cuvettes. These samples were then run under emission scan at manufacturer stated expected excitation (440 nm) were run across 460–600 nm excitation range in steps of 5 nm. Following both excitation and emissions scans on the NPS in DI water, tests were run on all other media utilized in the experimentation. These samples would ensure any solutions used with NPS would produce no distinct emissions from the same excitation wavelength to induce false positive intensity readings. Separate cuvettes were prepared with 3 ml of DI Water, 70% Ethanol and JM to detect the emissions from the determined ideal NPS Excitation wavelength. All media cuvettes were then run using the fluorescence excitation and emission range optimized for the NPS previously. There was then a separate run of a range of NPS concentrations matching the algal growth inhibition test suspended in JM (0–100 mg/l in steps of 10 mg/l). These samples were left in identical conditions to the Growth Inhibition Test (see Section 2.3.1) for 24 h before being examined to determine if NPS produces a linear intensity emission response to the concentration.

2.3. Algal exposure to NPS

2.3.1. Growth inhibition (GI) test

A testing procedure was produced by utilizing a modified version of the OECD for the Testing of Chemicals No. 201 (OECD, 2011). These runs would determine the growth rate averages the algae at each NPS exposure to determine the most visible sign detrimental impact of NPS to the algae. Eighteen 100 ml beakers containing 50,000 cells/ml of *R. subcapitata* suspended in JM were separated into triplicates. The first triplicate was a negative control group, then five further triplicates contained NPS at five different concentrations from 20 to 100 mg/l in 20 mg/l intervals from mixing in concentrate NPS solution (10,000 mg/l) into each beaker at specific volumes. All algal test cultures were then kept in the Incubator Shaker and maintained a constant 22 ± 2 °C temperature with a 75-rpm oscillation with a 16h/8h light/dark cycle to mimic sunlight hours during testing. The test was run over 72 h with checks every 24 h, with each beaker's algal cell concentration counted using the haemocytometer. This experiment was conducted twice with identical test set-up and regime, with the results being a combined average cell concentration per NPS exposure.

2.3.2. Confocal analysis

Confocal microscopy was conducted using a Zeiss LSM 510 Confocal Laser Scanning Microscope to analyse for any structural alterations in the *R. subcapitata* from NPS exposure, and to determine the location of the NPS on or within the algae from its fluorescence emission. The confocal laser was set to 458 nm with specific FITC filter to detect only emissions between 510 and 560 nm. Following GI tests, *R. subcapitata*

samples was prepped by pipetting 250 μl of media onto glass microscope slide and sealed with a cover slip. The confocal microscope was run to produce a two-part imaging analysis to provide imaging algal cells x50 and x63 (oil immersion) magnification. Analysis was firstly conducted using standard white light imaging for structural analysis followed by fluorescence imaging to analysis NPS positions. Imaging in fluorescence scans were also run at three different gain levels to assist defining exact NPS positions and intensity. Gains set to 600 ms (ms) were used to detect only very distinct fluorescing particle build-up to make clear separation from background fluorescence that came up in even control samples at higher gain. The 800 and 1000 ms gain images were used to demonstrate a clearer algal structure to define specific fluorescence positions and depths within or on the surface of the algal cell while still avoiding excess saturation of background emissions.

2.3.3. Algal UV–Vis fluorimetry (UV–Vis)

Further analysis was conducted on the UV/Vis fluorimeter to complement confocal analysis and determine if the NPS exposure to the algae caused a significant loss in fluorescence detection. Examinations for the fluorescence emission from the NPS was conducted with UV–Vis fluorescence analysis (Spectra-Max M Microplate Readers). The tests were run in unison with the confocal media set-up and used the same setup and media of NPS triplicates plus Jaworski controls. This potential reduction in fluorescence could result from algal uptake of the NPS, or from simple NPS agglomeration resulting in a loss of surface area for fluorescence emission. Prior UV–Vis analysis on the NPS would provide optimal excitation and emission range value. The examination would also analyse if *R. subcapitata* would present any emission from the same excitation value as the NPS. A 48-well culture plates were prepared and labelled with 4 cells per specific concentration of NPS (ranges of 10 mg/l steps from 0 to 100 mg/l NPS), with the control sample being made from blank JM. This was completed by taking each concentration triplicate and mixing the flasks by hand for 10 s. Then 4 ml of media was withdrawn by pipette from each beaker, before being combined in a single 25 ml glass beaker. Then this 12 ml of combined algal media per NPS concentration was pipetted evenly into the 4 pre-assigned wells in each culture plate at 0.5 ml media per well. An emission scan run at 440/505 nm Ex/Em determined from the prior UV–Vis fluorescence results with plate oscillation for 5 s prior to scan to ensure homogeneity. Each plate was run on a cross scan pattern (X), where the plates had 21 scans per well with 5 scans per arm of the cross. Following emission scans, the intensities values for all control and NPS exposed algae samples were averaged with standard deviation taken into account. Examination was focused on determining if the fluorescence intensity from algal exposed samples increased with matching linearity from pure NPS concentration vs intensity analysis. Examination primarily focused on whether intensity would decrease from a loss of NPS from uptake through the cell walls of the *R. subcapitata*. Surface agglomeration to algal cells and NPS self-agglomerating within an ion containing river replicate media could also cause mild loses in intensity as the surface area for fluorescence emission is decreased.

2.3.4. Raman analysis

Testing was conducted on *R. subcapitata* exposed to NPS using Raman analysis to examine if the polystyrene signals could be distinguished from the algal background. Test samples were chosen at random from each spare media used in the confocal testing. This enabled analysis of with algal samples in ranges from 20 to 100 mg/l NPS in 20 mg/l intervals plus Jaworski controls. A sample of 5 ml was added to separate labelled flasks. Then the media was oscillated by hand for 5 s before 1 ml of media was withdrawn by pipette from each flask and was placed in the well of a well glass slide. This slide was then dehydrated by leaving the slides in a controlled incubator at 50 °C for 30 min to remove the water while leaving the cells intact (examined under confocal imaging). Once on the slide the samples were placed under the Raman microscope white light imager at 60x magnification and focused in single *R. subcapitata*. X-maps were then run using the 532 nm laser at 60x magnification across the length of the algal cell. This analysis focused on the cell wall, as indication of NPS contamination had been found from confocal imaging (See Fig. 6). Several algal cells would be analysed per NPS exposure concentration and compared with control samples. To prevent the risk of thermal damage produced from the laser onto the algal cells, scans were conducted on algal cells in media and on dried samples. This would determine if it was possible to detect polystyrene signals through the Jaworski signal.

3. Results & discussion

3.1. Characterisation of NPS

3.1.1. Particle size confirmation and stability testing (DLS)

Analysis runs were conducted on 20 mg/l sample of NPS stored in DI water and JM over a 72-h period. The particle's diameter was analysed both to determine if the particles lose their 100 ± 10 nm expected size distribution, and to determine if this occurs from particle degradation or particle aggregation/agglomeration. Fig. 1 blue series spectra shows that even after only 24 h in DI water, there is a clear increase in distribution in the particle size outside of the 100 ± 10 nm stated diameter. For the sake of comparison to the JM, samples were determined to be "relatively" stable and within acceptable diameter between 80 and 120 nm. Following the 72 h of exposure to the experimental conditions, 62.3% of the NPS particles remained within this range. Fig. 1 also clearly demonstrated the distribution of particle size over the 72 h was distinctly changing in the >100 nm diameter. There has been a slight increase in the <100 nm diameter reading over 72 h, but it is clear the major alteration was the NPS are increasing in average diameter. This increase in average diameter represents a clear skew in favour of agglomeration of the particles. What the blue spectral data also shows is the process occurs from within the first 24 h of dilution in DI water, but only causes distinct changes after 72 h. The initial loss of stability in the first 24 h can be explained by the process of diffusion, in which the NPS surfactant is lightly stripped away into the DI water as the NPS was

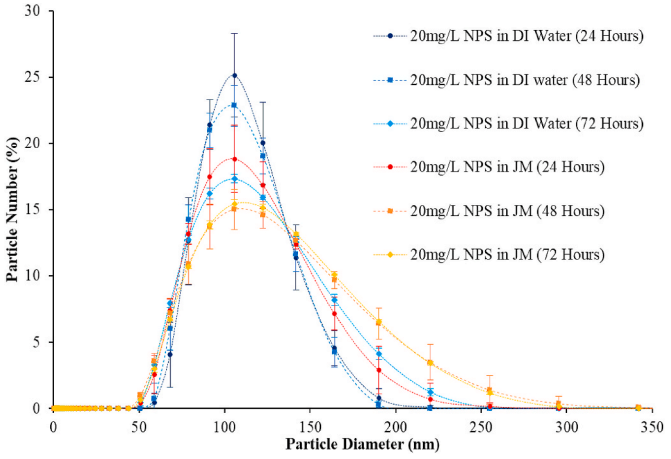

Fig. 1. DLS Spectra on 20 mg/l Nano-polystyrene Spheres (NPS) suspended within DI water (blue series) and JM (red series) under the conditions of the Growth Inhibition testing to determine the potential for aggregation under future experimental conditions. Samples ran across 72 h with checks on 24-h intervals demonstrating the gradual collapse in stability from a series of stresses that induced surfactant stripping. Initial particle stability was worse for JM with greater aggregation/agglomeration occurring in these samples compared to DI water exposure. This DLS response from NPS demonstrated that the ionic nature of the JM incurred notably more surfactant stripping over 72 h that seen in DI water samples, but all samples had a reduction of surface stability. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

diluted. The NPS particles were also kept in constant light oscillation and replicate daylight periods to represent the testing conditions they would later be exposed to. With the samples also being kept in constant oscillation, there is a natural mechanical weathering undergoing on the NPS surface as the water particles could scrub a certain amount of surfactant from the nano-spheres surface. A small amount of thermal ionization might also have occurred from the ambient heat and light-source, but the capacity for this to ionise/degrade the surfactant was likely negligible. This combination of surfactant diffusion, motion abrasion and minor excitation all increase the pressure on the surfactant to diffuse or degrade.

Over the 72 h these processes likely reduce the surface preservation efficiency of the surfactant, resulting in an increase of agglomerated NPS particles. Fig. 1 also presented the DLS results for NPS suspended within the JM (red spectra) used for algal culturing and acting as a replicate of river media. The vital purpose is determining whether agglomeration already demonstrated form various sources on NPS particles would be exacerbated by the addition of ionic compounds within the media. 20 mg/l NPS added into JM were tested with the identical conditions as samples analysed in DI water. The results present a clear change in response even after only 24 h, comparing to DI water samples there is a distinct change in NPS stability. Firstly, the peak diameter (ideally 100 nm, measured at 106 nm) within JM was only 15.7% of the overall NPS size distribution, compared to 25.1% when samples were in DI water. Secondly the NPS in Jaworski DLS spectra (red series) at every time interval have all undergone peak flattening and broadening that indicates a clear rise in particle stability reduction as samples increased or decreased from degradation of agglomeration. The key detail behind this curve flattening in all JM samples (Fig. 1 red series) is that the expected bell curve has a distinct right-side bias. The right-side bias is a direct sign the particles are aggregating/agglomerating, as the mean surface diameters are increasing to represent increasing levels of NPS particles clumping together. The overall results from Fig. 1 assessments demonstrated the NPS would already incur several sources of surface instability, however the results make it clear the Jaworski adds an additional level of surfactant removal. The additional source for this instability would be the presence of dissolved minerals and metals present in JM. The presence of ionic molecules in media, particularly with the addition of energetic sources like sunlight and oscillation, have been demonstrated to increase the surfaces that the surfactant will attempt to form agglomerates with (Hirano et al., 2017; Hotze et al., 2010; Laubie et al., 2013; Somasundaran and Cleverdon, 1985; Zhu et al., 2003). This surfactant removal into ionic solutions can be seen by how the initial 24-h collapse in stability remained more consistent by 72 h testing than the DI water samples. This indicates the ionic component was incurring the majority of instability, quickly removing the surfactant by ionic reactions that would reach dispersal equilibrium. Then the remaining stresses seen in DI water samples would continue to make slight increases in surfactant removal, making a minor further diameter instability. As such the protective layer of surfactant was being removed with this combination of initial reactive contaminants and then gradually with the prior stresses seen in DI water samples stripping. This indicated that samples of algae tested later might incur alternative effects from agglomerated/degraded NPS distinct from the pure particle, potentially more detrimental given the unstable surfaces.

3.1.2. *UV–visible fluorimetry (UV–Vis)*

The spectra analysis of emission ranges from various media (Fig. 2) demonstrated that the NPS particles produce a clear emission intensity absent from all other media. During the analysis the DI water, JM and JM containing 50,000 cells/ml *R. subcapitata* produced no distinct spectral response within the crucial range of 490–580 nm emission. Once each of these media were contaminated with 20 mg/l fluorescent NPS, the results were distinct. The addition of NPS to each of these media resulted in a consistent emission spectrum across the 490–580 nm values, with the only differences between media being the overall

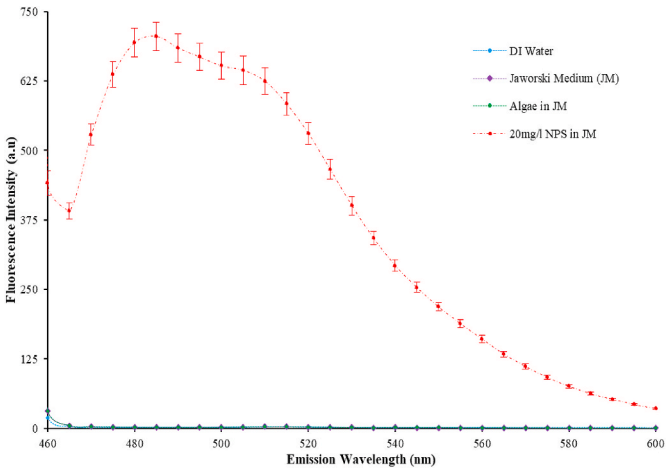

Fig. 2. UV–Vis fluorometric spectra on the two media samples utilized throughout R. subcapitata toxicity testing, de-ionised water and Jaworski. Samples of R. subcapitata and NPS were also suspended in these Jaworski medium to examine if any non-NPS source produced fluorescence emissions from the same 440 nm excitation wavelength of the nanoparticles. The spectra display the NPS remained completely distinct from any of the potential background emissions at 440 nm excitation wavelength of media or algal cultures.

intensity of each spectra. The alteration in overall intensity between media types was not clearly explained. However, the purpose of this experiment was to determine that NPS could be clearly identified within any media involved in testing, along with ensuring all test media had no distinct background from 440 nm excitation. The core determination was that no clear issues would be caused in reduction to the NPS emission from the media it is tested in. Similarly, the algae which the NPS would be tested against did not produce any notable intensities and thus would not produce any false positive results of media or algae giving off emissions from 440 nm excitation.

The results from Fig. 3 shows UV–Vis fluorometric readings produced a relatively linear rise in NPS emission intensity to concentrations in standardized NPS concentration range in JM. Once plotted to a trendline, it became clear the function was not perfectly linear, and was undergoing a consistent and continual minor reduction in emission intensity as concentration levels rose. The level of degradation to linear

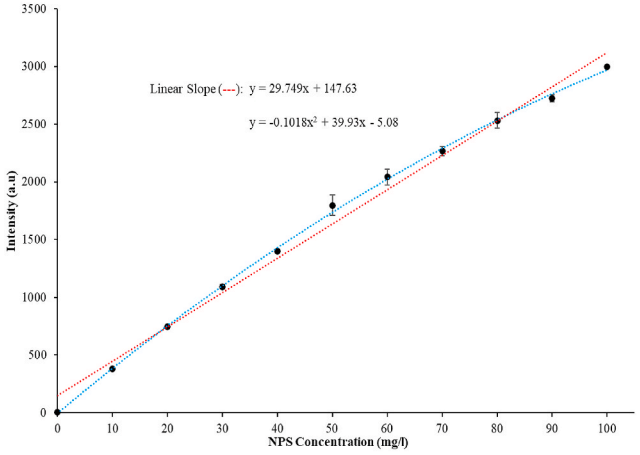

Fig. 3. UV–Vis intensity range spectrum of the NPS at a range of concentrations suspended in Jaworski media (JM) to determine if emission intensity was directly proportional to the concentration levels of the nanoparticle. The results show the emission intensity averages are relatively linear in response to NPS concentration, however there is a continual mild reduction in emission as concentration increases. This mild curvature can be considered for future intensity vs concentration assessment on algal samples.

intensity emission expectations throughout the testing remains quite low. Samples at the highest NPS exposure concentration (100 mg/l) presented intensity levels at only a 4.9% decrease in predicted linear value to optimal polynomial curve. Reviewing the results from DLS analysis in Section 3.1.1 it became clear that a small but notable portion of the NPS present in the media were expected to adhere and agglomerate in JM. However, the presence of this agglomerate was clearly not diminishing the fluorescence emissions from the NPS by any significant degree. The curvature and linear ideal slope are both relatively close to the uncertainty limits across all concentration of samples, but it is clear the graph supports the curved spectral position. As such the slight curvature is likely a representation of the limited amount of agglomerate NPS formation onto possible media debris (dust, etc.) or into agglomerate clumps. However, this minor emission reduction is not significant enough to indicate NPS absorption within algal cells or excessive agglomeration of NPS in media, which is further justified when compared to confocal results (See Fig. 5). As such the resulting spectrum showed that future tests on UV–Vis fluorimetry on NPS samples should present a relatively linear response between intensity of emissions to NPS concentrations. The other core determination was that emission intensity would incur deviation and uncertainties likely incurred from agglomeration that might present in the algal test as a consistent minor degradation in emission intensity.

3.2. Algal exposure to NPS

3.2.1. Growth inhibition (GI) test

The algal growth inhibition experiment was run twice, with Fig. 4 produced using the averaging of these two independent runs of the GI test. The overall analysis presented a clear association between NPS exposure levels and reduction with the *R. subcapitata* growth rate. However, it was also clear that even at the highest levels of NPS exposure the algae remain multiplicative. Over the 72 h of analysis, all algal growth rates had increased at a roughly exponential rate with a gradual reduction in the growth rate constant. From the control samples compared to the 100 mg/l NPS exposed algae, the growth rate had only reduced by 10.3%, which initially presents the NPS as quite non-hazardous. However, when the actual quantity of algae is checked after 72 h of NPS exposure, the cells/ml levels from control samples compared to the 100 mg/l NPS exposed algae had been reduced by 33.7%. The concentration of the *R. subcapitata* (C) were analysed to an exponential decay growth curve model:

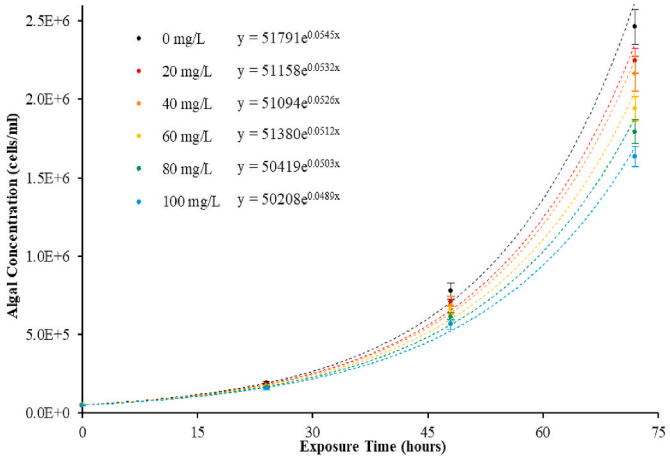

Fig. 4. Averaged rates of growth of the Raphidocelis subcapitata cultures over a 72-h period. The samples containing no NPS (0 mg/l) show more growth over each 24-h period than any polystyrene exposed samples. Similarly, the algal samples showed a steadily decreasing growth rate with every increase in NPS concentration, however there was no collapse in algal growth on any sample.

$$\mathbf{A} = \mathbf{A}_0(\mathbf{exp}^{kt})$$

Where (A_0) was the initial algal concentration at t = 0 and (*k*) corresponded to the growth rate constant of the specific algae when based on their NPS exposure concentration and concentration check time (t). As such should the growth rate from an algae exposed to a set concentration of NPS be reduced by a statistically significant degree compared to a control sample in pure JM, it would validate a conclusion that NPS were inducing toxic effects on the *R. subcapitata*.

These results demonstrated how even the seemingly relatively minor loss in rates from NPS exposure inflict clear losses in populations of algae after only a short period of time. The NPS material is thus causing a distinct impact on the capacity of algal cells to grow and develop, yet it is not inducing a culture collapse. This means it isn't easily discerned from the graph the impact on culture population to growth rate reduction, as seen by how even some of the 24 h samples already had population levels of algae notably impaired (19.17% concentration difference from control to 100 mg/l NPS exposed algae). As such another inference can be that the NPS induces the growth inhibition quickly, and after a longer period the amount of impairment kept rising but at reducing rates. The most likely conclusion is that NPS is an inhibitor in algal development but does not present acute toxicity. The NPS particles appear to be preventing either the development or replication processes of the algal cell while not inducing notable levels of algal death. Had the NPS proven toxic to *R. subcapitata*, the growth rates at higher concentrations of NPS exposure should have stagnated or even began to reduce in total concentration. This is clear as algal cultures are known for having a sensitivity to certain substances such as heavy metals or organo-halogens, with even low concentrations of these substances inducing population collapses (Expósito et al., 2017; Fu et al., 2017; Horvatić et al., 2007; Kumar et al., 2014; Kusk et al., 2018; Lee and Chen, 2009). Additionally, the DLS results produced from Fig. 1 already indicate the strong possibility that the NPS will have become surface reactive and prone to agglomeration. This provides the possibility that NPS could induce a chronic toxicity effect at lower NPS concentrations over longer periods of time, however the test model utilized only focused on acute toxicity effects. To determine how the NPS induces growth inhibition yet non-acute toxicity on algae, confocal microscopic analysis would examine where the NPS contaminated the algal cell, by agglomerating to the wall or permeates into the cell organelles. The crucial conclusion so far is that the NPS retain a capacity to inhibit the algal cell growth by some means, with even lower concentrations producing discernible if small reductions.

3.2.2. Confocal analysis

Analysis of the *R. subcapitata* cells exposed to various levels of NPS proved extremely useful in identifying a likely cause behind the growth rate reduction. Fig. 5 was a representative sample of the results from control algal cells when tested using the white light and 458 nm laser excitation imaging. It became clear that the algal cells produce their own inherent emission between 510 and 560 nm from the laser excitation. These fluorescence emissions consistently aligned with the structure of the algal cell seen from white light images taken previously. The emissions from the algae in fluorescence imaging remains faint but quite distinct compared to the image background. The imaging also demonstrates that the fluorescence emissions from the algae is relatively uniform across the cell, with slight increase in intensity near the cell walls and reduced intensity from the cell walls. Also noticeable was that all cells analysed remained within expected cell diameter ranges and surface structure, and no signs of notable cell death. As seen in Fig. 5, initial white light imaging would indicate no alteration, no clear morphological changes from the resulting polystyrene exposure. However, upon examination of NPS exposed samples at even the lowest exposure concentration there was a noticeable change in the fluorescence images. From the 20 mg/l NPS samples onward, the fluorescence imaging

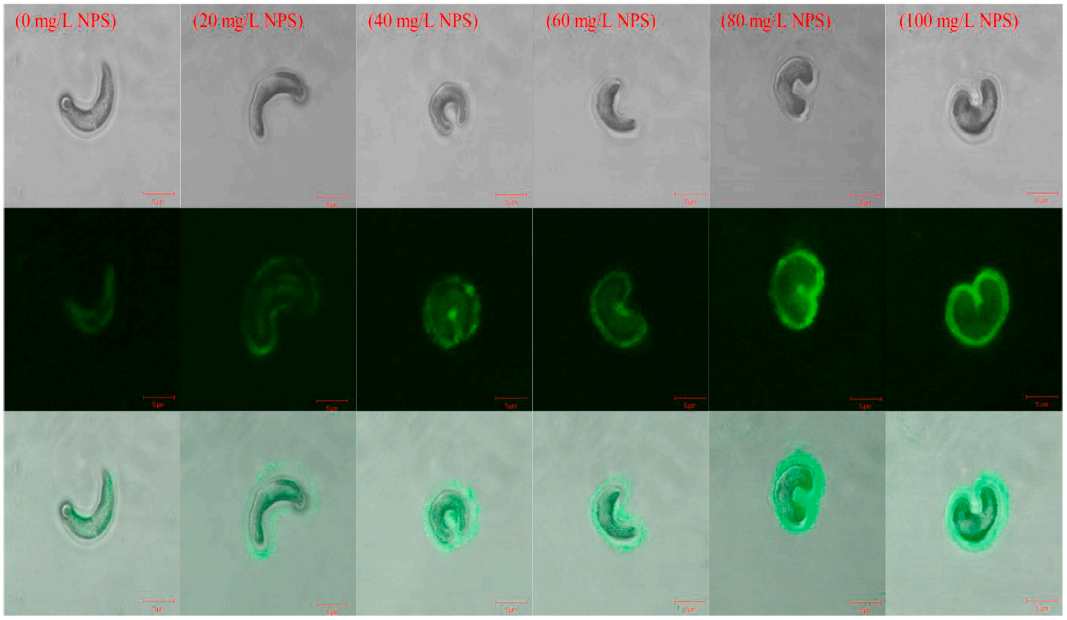

Fig. 5. Confocal imaging samples representative of R. subcapitata exposed to a range of NPS concentrations. The columns demonstrate the change in NPS exposure concentration (as labelled on top in red), while the rows show the white-light (top), epifluorescence (middle) and combined imaging (bottom). As the concentration of NPS increased, there is a clear increase in fluorescence intensity around the cell walls of the algae that is distinctly not present in control (0 mg/l NPS) samples. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

showed the expected *R. subcapitata* shape, with a very minor background emission as seen in control cells. However, an additional distinct fluorescence was present, a layer of more prominent fluorescence that appeared to surround the algal cell. In certain lower NPS exposure concentrations this fluorescence "coating" could appear fragmented, with gaps in the layer or positions with reduced emission intensity. These layers were not seen in control samples, either from physical observation during fluorescence analysis, or in later sample images taken. This coating surrounding the cell walls were most likely NPS particles which had agglomerated onto the cell walls and/or forming an agglomerate around the algae. The apparent volume and uniformity of the coating, based on the emission intensities demonstrated, were seen to increase in direct relation to the NPS exposure concentration. Samples of 20 mg/l NPS exposure present thin coatings with numerous disruptions in the cohesion of the layer, while samples at 60 mg/l NPS exposure demonstrate virtually uniform and relatively concentrated layers around the cell walls. Once *R. subcapitata* cells were exposed to 80–100 mg/l NPS levels, the NPS coating was significantly dense and produced emissions that appeared to dampen those from the algal cell itself. Imaging also indicated that the inherent emissions from the algal cells themselves was not notably altered throughout the NPS exposure levels, although this cannot be guaranteed.

This effect has already been seen from other nanomaterials in research with *R. subcapitata*, further validating the scenario of NPS cell wall adherence from the loss of the surfactant and increased NPS surface ionization (Bhattacharya et al., 2010; Huarachi-Olivera et al., 2019; Mao et al., 2018; Ribeiro et al., 2019; Yenigün, 2019). The data found from this testing and compared to the growth inhibition experimental results (See Fig. 4) presents the possible impact from the polystyrene. It appears that at least under short term exposure, the plastics are not absorbed by the algae in significant levels. Crucially there was no notable visual signs of increasing cell death or physical malformations compared to control *R. subcapitata* cells at any NPS exposure concentration. The nanoparticles are instead agglomerating to each other whilst binding to the algal cell walls from their surface instability. The most likely result would be a reduced ability for the algae to either cell divide and reproduce, along with blocking possible pathways for minerals and water to sustain the cells. This assumption is also supported from the confocal imaging relating to the rise of NPS concentration to the consistency and depth of the agglomerate NPS on the algal cells. Growth inhibition (Fig. 4) comparisons to confocal imaging display a direct correlation to the NPS coating on the algae. Initially the NPS became surface reactive and thus incurred increased agglomeration, and these surface reactive NPS thus became more cohesive and formed patchwork agglomerates across the algal cell wall as seen in Fig. 5. As discussed previously, these algal-bound agglomerates were known to reduce the capability for nutrition absorption and overall population growth (Bergami et al., 2017; Bhattacharya et al., 2010; Nolte et al., 2017; Zhang et al., 2017). Once the concentration reached 60 mg/l NPS, algal cells were completely coated and would now only become more densely layered in NPS. When compared to the growth inhibition results (Fig. 4) it was clear there was a deviation in response from >40 mg/l NPS exposure algal compared to the <40 mg/L NPS exposed algal. It is clear algal cells can continue to reproduce over all NPS exposure concentration, but their rates began to enter a significantly reduced state once NPS exposure concentration were over 40 mg/L. Whether this was due primarily to nutrition loss or increased strain on cell division is not clear from the imaging analysis.

3.2.3. *Algal UV–Visible spectroscopy (UV–Vis)*

Analysis on *R. subcapitata* exposed to a concentration range of NPS particles showed a clear linear rise in emission intensity from 440/505 nm Ex/Em the rising concentrations of NPS (Fig. 5). These samples had been left in the media for 24 h at the same settings as algal growth inhibition tests would be conducted (light, temperature, etc.) prior to analysis to ensure the NPS was representative of tests samples. The resulting spectrum was also compared to the slight non-linear regression response of emission intensity to increased levels of NPS seen in the control tests (Fig. 2). The examination of the prior NPS spectrum demonstrated that the expectation of uncertainty from agglomeration was not a gradual reduction in intensity but an arbitrary variation in the intensities. The confocal imaging seen in Fig. 4 also suggested the algal cells were not absorbing any discernible levels of NPS through the cell wall, as such emission intensity would not be expected to be lost from algal particle uptake. The UV–Vis spectrum produced from algae exposed to NPS (Fig. 6) clearly complements the confocal image

conclusion, as the intensities were still proportionate to their exposure concentration. Firstly, the original model demonstrated a very mild regression curve in emission intensity as NPS concentration increase, while the analysis of algae exposed to NPS at increasing concentrations in Fig. 6 provides a clear linear response.

The second key alteration in NPS-algae results from the control tests was the emission intensity value. As all settings had been kept consistent from Growth Inhibition testing and the NPS exposure concentrations are the same range (0–100 mg/l in JM) the intensities should have been relatively identical (taking result deviation into account). The intensities emitted from the NPS in algal exposure samples were on average reduced by 51.7% from control NPS samples (See Supplemental Table 1). The most likely cause behind both the linearity response and the intensity difference when comparing algal exposed NPS to the NPS control tests was the NPS agglomeration. In short, NPS in JM seen in DLS (Fig. 1 red series) and confocal Imaging (Fig. 5) caused agglomeration, reducing the overall surface area of the NPS and thus reducing the overall emission intensities. This was seen from the gradual reducing of emission in the UV–Vis of these particles, with the agglomeration impacting the higher NPS concentrations more as higher concentrations led to easier agglomeration (Allouni et al., 2009; Bruinink et al., 2015; Hollander et al., 2002; Yang et al., 2012). This general ~50% reduction in overall comparative intensities from NPS control samples seen in Fig. 3 does make sense considering the DLS and confocal imaging information demonstrated the NPS were agglomerating. The NPS were undergoing several forms of agglomeration that resulted in surface area reduction such as their surfactant diffusion into media, the instability from interaction with ionic components in the JM, and from agglomerating to the algal cell walls. The aggregation/agglomeration of a particle will inherently reduce the overall reactive surface area of the NPS at the same concentration, thus reducing surface reactions and effects (Fu et al., 2014; Halappanavar et al., 2019; Lee et al., 2013; Lehtiniemi et al., 2018; Suchomel et al., 2018).

As such the particle's fluorescence emission, which only emit from available surface area, will also be reduced in overall intensity as the surface area reduces. Overall, the intensity reduction can be attributed to the collapse in NPS average emissive surface area from the numerous sources of agglomeration. Following this overall emission intensity reduction there is the change in individual emission response, with the emission reduction becoming less dramatic as NPS concentration rose (Supplemental Table 1). Since the quantity of algal cells in each test media are identical (50,000 cells/ml), the quantity of potential agglomeration surface is identical across all tests. While the confocal imaging results (Fig. 5) show the NPS agglomerate layer on the algae does increase with NPS concentration, it is unclear if rate of NPS agglomerating and losing surface area increased with the rise in NPS concentration. Confocal imaging already demonstrated that the NPS almost completely covered the available algal surface by 40 mg/l. Once this surface was coated with NPS exposed algae, the formation of a thicker NPS agglomerate layer were seen to form in >40 mg/l NPS exposed algae in Fig. 5. It is possible that, while at lower NPS exposure concentrations the confocal showed lighter NPS agglomeration onto algal cells, the proportion of overall NPS in the media agglomerated like this was proportionally higher than the higher concentration samples. In short it appears increasing NPS concentrations simply increase the likelihood of surface reactive NPS colliding and forming thicker layers of agglomerates in the media or on algal cell walls.

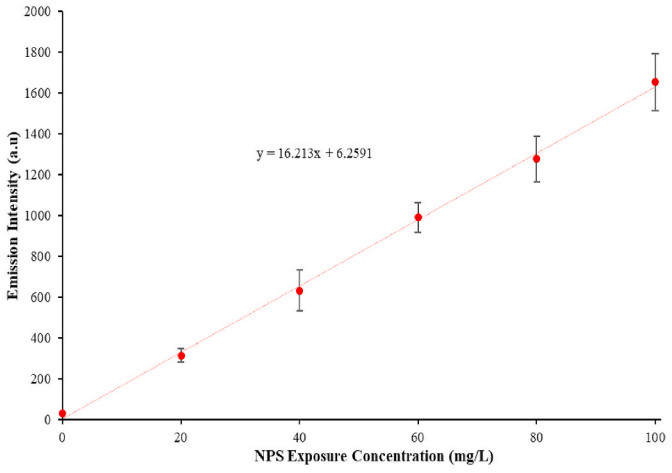

Fig. 6. UV–Vis fluorescence intensity values for a fixed concentration of R. subcapitata exposed to a range of NPS concentrations after 24 h exposure. The results remain linear and within deviation variables across the NPS range, demonstrating an expected response from the consistent increases in NPS exposure concentrations.

3.2.4. Raman analysis

Fig. 7 presents the Raman spectra of several samples of *R. subcapitata* samples dried out from media and imaged on glass slides based on their NPS exposure concentration. Each concentration spectrum presented in Fig. 7 were comprised by averaging the emission signals from six individual scans on R. subcapitata cell samples at matching NPS exposure concentration. The spectra all present expected features from algal cells, with the clear carotenoid peak from the 1527 cm^{-1} peak that would be present in any plant cell structure (Jehlicka et al., 2019; Velitchkova, 2014). Another common carotenoid that functions as a chlorophyll protector in many plant and algae cells was also expected between 1150–1157 cm^{-1} (Gall et al., 2015; Parab and Tomar, 2012). The analysis was focused on determining polystyrene presence either within or on the surface of the algal cells. Under Raman analysis there is one key peak likely to be present from the polystyrene at approximately 1001 cm^{-1} which relates to the C═C breathing mode vibration in the styrene chain (Domratcheva-lvova et al., 2017; Gillibert et al., 2019; McCreery Research Group, 2014). However, analysis of the spectrum makes it clear there is an existing peak seen around the 1006 cm^{-1} wavenumber in NPS and control *R. subcapitata* samples that clearly comes from the algal cell. Literature research indicated the 1006 cm^{-1} peak is an expected emission corresponding to another carotenoid signal that has been seen in other Raman tests on algae (Jehlička et al., 2014; Osterrothová et al., 2019). The sharp and intense emission from this

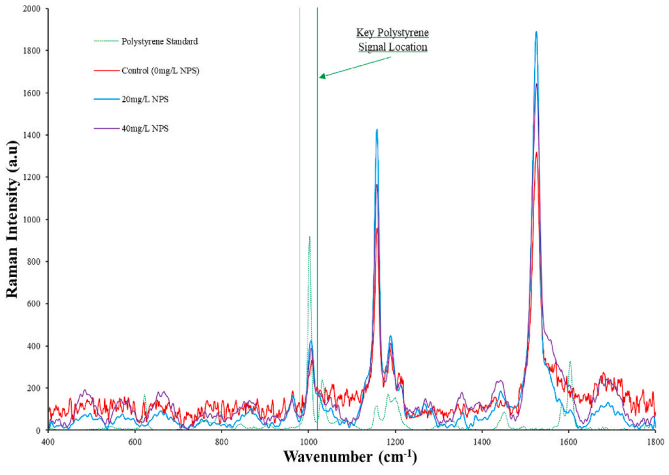

Fig. 7. Raman spectra of R. subcapitata cell wall boundaries with varying levels of NPS concentration exposure along with a pure polystyrene control sheet. Analysis shows the crucial peak needed to discern polystyrene presence (1001 cm^{-1}) has an existing peak from a carotenoid band from the chlorophyll pigment, also seen at ~1150 cm^{-1} and ~1520 cm^{-1}. This issue was also exacerbated by the overall algal background preventing discernible details of the any polystyrene peak.

peak appears to completely swamp any emission that might be released by the NPS key 1001 cm^{-1} polystyrene marker peak.

Similar to this issue, any additional peaks known from polystyrene (621 cm^{-1}, 1031 cm^{-1} & 1583 cm^{-1}) are all distinctly weaker in intensity than the 1001 cm^{-1} peak and are liable to be lost in the emission background of algal samples. The examination of all spectra individually failed to present any distinguishable peak either within algal cells or on the cell walls. There was a potential that the constant oscillation in media followed by thermal drying might have been enough to dislodge some NPS agglomerated to the cell wall surface. Similarly scans inside the algal cell were initially conducted but reached the same issues, along with the confocal imaging indicating NPS was not expected to be present within the cell (Fig. 5). Attempts were made to analyse samples within a shallow amount of JM to prevent possible processing issues, but accuracy from the laser through even the fine layer of media were severely reduced. The presence of the water layer continued blocking a lot of the emissions, only to be exacerbated by emissions from the media ions, minerals and aquatic debris from dust or algal remains all prevented any accurate assessment of the algal cells. There remained a potential for Raman to prove accurate results with more processing steps and better dehydration to reduce the influence of algal carotenoids and preventing NPS loss from the cell walls.

3.3. General discussion

The central finding from our research was that NPS inflicted a distinctly detrimental yet non-catastrophic impact onto *R. subcapitata* algae, with evidence indicating this was primarily due to the surface agglomeration of the NPS particles. Analysis on growth rate in Section 3.2.1 showed that whilst the NPS did not induce culture collapse at even 100 mg/l exposure, there were clear statistical reductions in the growth rates on all NPS exposure tests compared to control samples. The examination showed that by 100 mg/l NPS exposure after 72 h the algae had suffered a 33.7% drop in number of algal cells compared to control samples. The reduction across NPS samples was enough to indicate that the NPS were inducing a level of stress even at 20 mg/l NPS exposure that could impact their rate of growth. Once examined under fluorescence imaging in Section 3.2.2, it became clear the issue related strongly to the presence of NPS agglomerates that were forming surface coating layers on the *R. subcapitata*. Examination makes it clear that the NPS were potentially preventing the growth capabilities of the algae by both physically preventing the cell fragmentation and by limiting the space for nutrient uptake. Additionally, any reactive ions bound to the agglomerate NPS would now be in direct and continuous contact with the algal cell walls, inducing a further level of damage and stress on the cell. This agglomeration was already detected throughout Section 3.1 in the DLS analysis of the NPS independently in the Jaworski Medium, where the minerals and substances present can ionise and become free radicals. While this combined effect of NPS agglomeration and surface contamination do not induce a culture collapse, their combined effect must be noted for the potential of long-term damage. The NPS agglomerates adhered to algal cells despite continual motion within their incubator, and so are clearly resilient to removal by the most common source of contamination clearance. The only likely solution would be an immediate removal of NPS from the media and a long period of cell reproduction in fresh media to slowly diffuse and disperse over time. Should algae become tainted by similar surface reactive micro/nano-plastics they would likely incur the same effect and thus remain contaminated for a long period of time. Given the calculated growth rate reductions seen in our samples, which were kept in optimal growing conditions, the presence of NPS in an actual freshwater body over time could seriously hinder the development of the algae. These issues on the health of *R subcapitata* specifically don't even take into account the fact that algae are a crucial primary food-source for aquatic herbivores, who are in turn crucial for the predators to feed on.

Once these issues are contextualised for algae in the environment there are two clear risks, with the first being the obvious issue of NPS or similar MNPs reducing the growth rates and thus the population size of algae. The potential for gradual algal decline from our research is clear and could be significant over the long term, and once severely diminished the remaining creatures in the food-chain would also suffer the starvation effects. This risk from MNP is not new, other algae and plankton exposed to micro/nano-plastics have already similarly shown indications of growth reduction and potential culture collapse (Botterell et al., 2019; Figueiredo and Vianna, 2018; Frias et al., 2014; Nolte et al., 2017; Setälä et al., 2014; Sjollema et al., 2016). This possibility would require a sustained nano-plastic contamination at appreciable levels to the existing test concentrations, however the occurrence and persistence from degraded bulk plastics entering micro/nanoscale has been readily shown across the environment (Ballent et al., 2016; Blettler et al., 2018; da Costa et al., 2016; Halstead et al., 2018; Sutton et al., 2016; Wang and Wang, 2018; Windsor et al., 2019; Zhao et al., 2019). The second and far more uncertain risk is that the surface agglomerated MNPs might never reach concentrations to cause algal culture collapse but will be ingested by algal predators and be passed up the food-chain. This bottom-up contamination of a primary food-sources like algae with micro/nano-plastics being subsequently passed up and concentrated in organisms higher in the food-chain has already been demonstrated in existing research (Dehaut et al., 2019, 2016; Santana et al., 2017; Toussaint et al., 2019). Algae might remain healthy enough to maintain quantities capable of sustaining their eco-systems despite MNP contamination, yet the chronic impact on other species up the food-chain is unpredictable and potentially disastrous. Eventual transitions into creatures consumed by humans would be inevitable, and thus the risk to all people being exposed and ingesting micro/nano-plastics that may also be surface contaminated becomes a clear threat (Bouwmeester et al., 2015; Galloway, 2015; Hitchcock and Mitrovic, 2019; Joon, 2019; Smith et al., 2018). As such while the acute toxicity test might indicate the NPS needed quite high concentrations to be "acutely" toxic, the OECD acute toxicity model should also recommend optional assessments to study the risks from continual contamination when dealing with MNPs. These "transitional" assessments on substances in sub-micron sizes to acute toxicity tests are already being advised by other researchers (Chae and An, 2017; Everaert et al., 2018; Gallo et al., 2018). This would enable researchers to use acute toxicity tests for rapid assessment of toxicity risks while providing initial evidence for whether a chronic focused test was required in cases where the MNP remained persistence on or within the test organisms, posing a continual risk within an eco-system. With these additional assessments, the acute toxicity analysis can remain vital as an initial preventative action to predict micro/nano-plastic detrimental effects both acute and chronic.

4. Conclusions

Through the application of a standardized acute toxicity test along with additional analytic techniques, our research showed that on *R. subcapitata* algae exposed over 72 h to a range of concentration of 100 nm polystyrene spheres (NPS) induced growth inhibition up to 33.7%. Additional techniques such as Dynamic Light Scattering (DLS) and confocal Microscopy presented evidence that this growth inhibition was primarily the result of NPS agglomerating within media. This NPS agglomerate became adhered to the algal cell walls, diminishing the nutrient uptake and cellular reproduction of the algae. These results adds to the growing body of research which indicated that micro/nano-plastics (MNPs) within the environment could be both a cause for catastrophic population reductions to algal species while also acting as a lingering contamination on surviving algal cells, which in turn would have knock-on impacts on the species that rely on algae for sustenance.

CRediT author statement

Andrew Reynolds: Conceptualization, Methodology, Investigation,

Writing – Original Draft, Writing – Review & Editing, Visualization. Michelle Giltrap: Conceptualization, Methodology, Resources, Supervision. Gordon Chambers: Conceptualization, Resources, Supervision, Funding Acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecoenv.2020.111153.

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